Prevention of chronic kidney disease and its consequences

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RELEVANCE OF CORRECTION FOR DRIFT AND DAY-TO-DAY VARIATION IN CYSTATIN C MEASUREMENT:

A POST-HOC ANALYSIS OF THE PREVEND COHORT, WITH INDEPENDENT REPLICATION IN THE ESTHER COHORT

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Abstract

Despite standard laboratory quality control, drift and day-to-day variability in cystatin C measurements can be observed. We investigated whether correction for drift and day-to-day variation in cystatin C measurements improves the association of estimated glomerular filtration rate (eGFR) with Chronic Kidney Disease (CKD) risk factors and prognosis. Plasma samples of the PREVEND study (Dutch cohort study, n=8,592) were used to measure cystatin C (Gentian assay) on 243 random days. A correction factor was calculated for each measurement day. GFR was estimated with CKD-EPI equation using routinely measured cystatin C ($eGFR_{cysC}$) and corrected cystatin C ($eGFR_{cysC\ corr}$). Participants were categorized in 6 categories of $eGFR_{cysC}$ and $eGFR_{cysC\ corr}$: ≥120, 90–119, 75–89, 60–74, 45–59 and <45 ml/min/1.73 m^2. Independent replication was performed in the ESTHER study (German cohort study, n=9,949). Compared to non-reclassified participants, participants re-classified upward had significantly lower age, body mass index, blood pressure, cholesterol, glucose and albuminuria, whereas the opposite was true for participants reclassified downward. CKD risk factors explained more variance in $eGFR_{cysC\ corr}$ than in $eGFR_{cysC}$ ($p < 0.001$). Compared to non-reclassified participants, risk of incident cardiovascular events (n=789, follow-up 9.3 ± 2.7 years) tended to be higher in downward reclassified and lower in upward reclassified participants. Net reclassification improvement for incident cardiovascular events using $eGFR_{cysC\ corr}$ was positive (0.102, p=0.019). The ESTHER study showed similar results. Correction for drift and day-to-day variation in cystatin C measurement improves eGFR using cystatin C for its association with CKD risk factors and incident cardiovascular events.

INTRODUCTION

Impaired kidney function is associated with various adverse health outcomes, such as progression to end-stage renal disease, cardiovascular events and mortality (1-3). Kidney function is routinely assessed in medical practice by measuring filtration markers and imputing the obtained values in equations to estimate glomerular filtration rate (eGFR) (4). Variability in measurement of these filtration markers leads to imprecision (5), resulting in misclassification of individuals in eGFR categories.

Recently cystatin C has been suggested as a better marker of kidney function than creatinine (6-9). Although the performance of cystatin C to estimate GFR has not been definitely established yet, it is clear that eGFR estimated from cystatin C is more consistently associated with incident renal failure, cardiovascular events and all-cause mortality than eGFR estimated from creatinine (7, 10). Recently, the validity of the measurement of cystatin C has been improved by the release of international certified reference material for calibrating laboratory assays (11, 12). However, despite the use of such reference material and standard laboratory quality control, drift and day-to-day variability in cystatin C measurements can be observed.

For epidemiological research as well as clinical care, it is important to obtain the most reliable measurement of filtration markers to estimate GFR. Therefore, we investigated whether correction for drift and day-to-day variability in cystatin C measurement would improve the association of cystatin C based eGFR with chronic kidney disease (CKD) risk factors and prognosis.

METHODS

Study population and design

For this study, data of participants of the Prevention of Renal and Vascular End-stage Disease (PREVEND) study were used. The PREVEND study is a prospective cohort study designed to investigate the natural course of albuminuria and its relation to renal and cardiovascular disease in a large cohort drawn from the general population. Details of the study protocol have been published earlier (13, 14). In summary, during 1997-1998, all inhabitants of the city of Groningen, the Netherlands, aged 28 to 75 years (n=85,421) were sent a 1-page postal questionnaire regarding demographics, disease status (existing and history), life style habits, use of medication, and pregnancy, and a vial to collect a first morning void urine sample. A total of 40,856 (47.8%) individuals responded. Pregnant women (defined by self-report) (n=283) and subjects with insulin-dependent diabetes mellitus (defined as requiring the use of insulin) (n=379) were excluded. After the additional exclusion of individuals who were unable or unwilling to participate in the study, individuals with a urinary albumin concentration of 10 mg/L or greater

(n=6000) and a random sample of individuals with a urinary albumin concentration of less than 10 mg/L (n=2,592) completed the screening protocol and formed the baseline PREVEND cohort (n=8,592).

For the current analysis participants with missing data on cystatin C (n=562) were excluded, leaving a total of 8,030 participants. The PREVEND study has been approved by the local medical ethics committee and is conducted in accordance with the guidelines of the Declaration of Helsinki.

Measurements

Blood samples were obtained from all participants. Serum cystatin C levels were measured routinely from baseline blood samples that were stored frozen at -80° Celsius until assessment (Cystatin C PETIA assay, Gentian, Moss, Norway), on a Roche Modular auto-analyzer (Almere, the Netherlands). Measurements took place on 243 random days between March 2010 and October 2012 in the Clinical Chemistry Laboratory at the University Medical Centre Groningen, Groningen, the Netherlands. Cystatin C measurements were reported as milligrams per litre (mg/L). Standard laboratory quality control, in accordance with the advice of the manufacturer, included monthly recalibration of the analyzer and a daily check of 2 reference materials provided by Gentian and recalibrating the analyzer in case the obtained value was more than 3 standard deviations (0.02 mg/L for the lower and 0.08 mg/L for the higher range) above or below the reference value. At each measurement day, also two samples of same plasma pool were included to act as “reference samples”. Plasma pool samples were stored at -80° Celsius and were defrosted immediately before use. Cystatin C concentration was measured in this plasma pool and calibrated against the international standard for cystatin C (11, 12). Subsequently, for each measurement day a correction factor was calculated to adjust for drift and day-to-day variation in cystatin C measurement: calibrated cystatin C concentration in the plasma pool divided by the mean cystatin C concentration that was actually measured in the two plasma pool samples that specific day. This correction factor was used to adjust all cystatin C values measured that day. Cystatin C concentration in the plasma pool samples calibrated against the international standard was 0.71 mg/L. Using the cystatin C based CKD-EPI equation (6), GFR was estimated from routinely measured cystatin C concentration (i.e. $eGFR_{cysC}$) and also from cystatin C corrected for drift and day-to-day variation in measurement (i.e. $eGFR_{cysC\ corr}$).

Total cholesterol and plasma glucose were measured by dry chemistry (Eastman Kodak, Rochester, New York). Participants collected two 24-hour urine samples, in which urinary albumin concentration was determined by nephelometry (Dade Behring Diagnostic, Marburg, Germany). Urinary albumin excretion (UAE) was calculated as the mean of two 24-hour urine collections. Age, sex, race and smoking were assessed by questionnaire. Smokers were classified as current smokers if they were currently smoking or stopped smoking less than one year ago.

Weight was measured at the outpatient clinic to the nearest 0.5 kg with a Seca balance scale (Seca Vogel & Halke GmbH & Co, Hamburg, Germany). Height was measured to the nearest 0.5 cm. Blood pressure was measured on two occasions in supine position on the right arm every minute for 10 min, with an automatic Dinamap XL model 9300 series monitor (Johnson-Johnson Medical Inc., Tampa, FL, USA). Blood pressure was calculated as the mean of the last two measurements at both occasions.

Incident cardiovascular (CV) events

Incident CV events were defined as incidence of fatal and non-fatal myocardial infarction, stroke, ischemic heart disease, revascularization procedures or cardiovascular mortality. Data for mortality and cause of death were received from the Dutch Central Bureau for Statistics. Information for hospitalization for CV morbidity was obtained from PRISMANT (Utrecht, the Netherlands), the Dutch national registry of hospital discharge diagnoses. All data were coded according to the International Classification of Diseases, 10th Revision and the classification of interventions.

Statistical analysis

Data are presented as mean ± standard deviation or median (inter-quartile range) for continuous variables and number (percentage) for categorical variables. First, study characteristics were calculated for the overall population and according to categories of $eGFR_{cysC\ corr}$. Second, the associations of $eGFR_{cysC}$ and $eGFR_{cysC\ corr}$ with CKD risk factors (i.e. age, sex, systolic blood pressure, blood glucose, total cholesterol, body mass index, UAE and smoking) were evaluated using univariate and multivariate linear regression analyses. Third, model discrimination was assessed by bootstrapping the difference in adjusted R-square of two multivariate linear regression models (15). Fourth, to assess reclassification, we created a 6×6 cross-tabulation of the $eGFR_{cysC}$ and $eGFR_{cysC\ corr}$ categories where eGFR was categorized using the following cut-offs: ≥120, 90-119, 75-89, 60-74, 45-59 and <45 mL/min/1.73 m^2. Participants with eGFR <30 and <15 mL/min/1.73 m^2 (CKD stages 4 and 5) were not considered separately in the present study because there were few participants in these categories (n =19 for $eGFR_{cysC\ corr}$ and n=17 for $eGFR_{cysC}$). We calculated the proportion of participants reclassified using $eGFR_{cysC\ corr}$ in each category of $eGFR_{cysC}$. We assessed whether CKD risk factors differed between participants reclassified and those not reclassified. We evaluated risk of incident CV events in reclassified participants using Cox proportional hazards models. Lastly, to further evaluate improvement in reclassification, we calculated net reclassification improvement (NRI) for incident CV events (16).

Replication study

There might be laboratory and/or assay specific reasons for drift and day-to-day variability in cystatin C measurements and consequently for reclassification across eGFR categories. Therefore, we also investigated in another, independent prospective cohort study (the ESTHER study) (17) to see whether correction for drift and day-to-day variability in cystatin C measurement improved the association of cystatin C based eGFR with CKD risk factors and prognosis. A detailed description of the ESTHER study design is given in Appendix Text. This study is, like the PREVEND study, general population-based and included 9,949 participants from the federal state of Saarland in Germany. For this study, cystatin C concentration was measured applying standard laboratory quality control on 93 random days between November 2005 and February 2007 from baseline blood samples. Baseline blood samples were stored frozen at -80° Celsius and were defrosted immediately before use (Cystatin C PENIA assay, Siemens, Marburg, Germany). Measurements were performed in the Biomarker Laboratory of the Department of Internal Medicine II-Cardiology at the University of Ulm Medical Centre. Standard laboratory quality control included a daily check of 2 reference materials and recalibrating the analyzer in case the obtained value was more than 3 standard deviations (0.03 mg/L for the lower and 0.05 mg/L for the higher range) above or below the reference value. As provided by Siemens (manufacturer), cystatin C concentration in lower reference material was 0.89 mg/L and in higher reference material was 1.76 mg/L. At each measurement day, these two reference samples were measured and used to calculate day-specific correction factors to adjust routinely measured cystatin C concentration for drift and day-to-day variability in measurement. Similar statistical analyses were performed as described above. Additionally, in regression analysis, it was tested whether both concentrations of reference material (the lower and the higher reference cystatin C concentration) show similar fluctuation in cystatin C measurement.

RESULTS

Study Characteristics

Characteristics of PREVEND study participants overall and in 6 categories of $eGFR_{cysC\ corr}$ are shown in Table 1. Participants in lower categories $eGFR_{cysC\ corr}$ were more likely to be older, male, and to have higher levels of blood glucose, systolic blood pressure, body mass index, UAE and total cholesterol compared with individuals with $eGFR_{cysC\ corr}$ of 90-119 mL/min/1.73 m^2. Mean $eGFR_{cysC\ corr}$ was higher than mean $eGFR_{cysC}$ overall (94.9 ± 19.1 and 92.8 ± 19.1 mL/min/1.73 m^2, respectively; $p < 0.001$), as well as across all categories of $eGFR_{cysC\ corr}$. Figure 1 shows drift and day-to-day variability in cystatin C concentration in plasma pool samples that occurred during the measurement period that comprised 243 days.

Figure 1 | **Drift and day-to-day variability in cystatin C concentration in plasma pool samples**

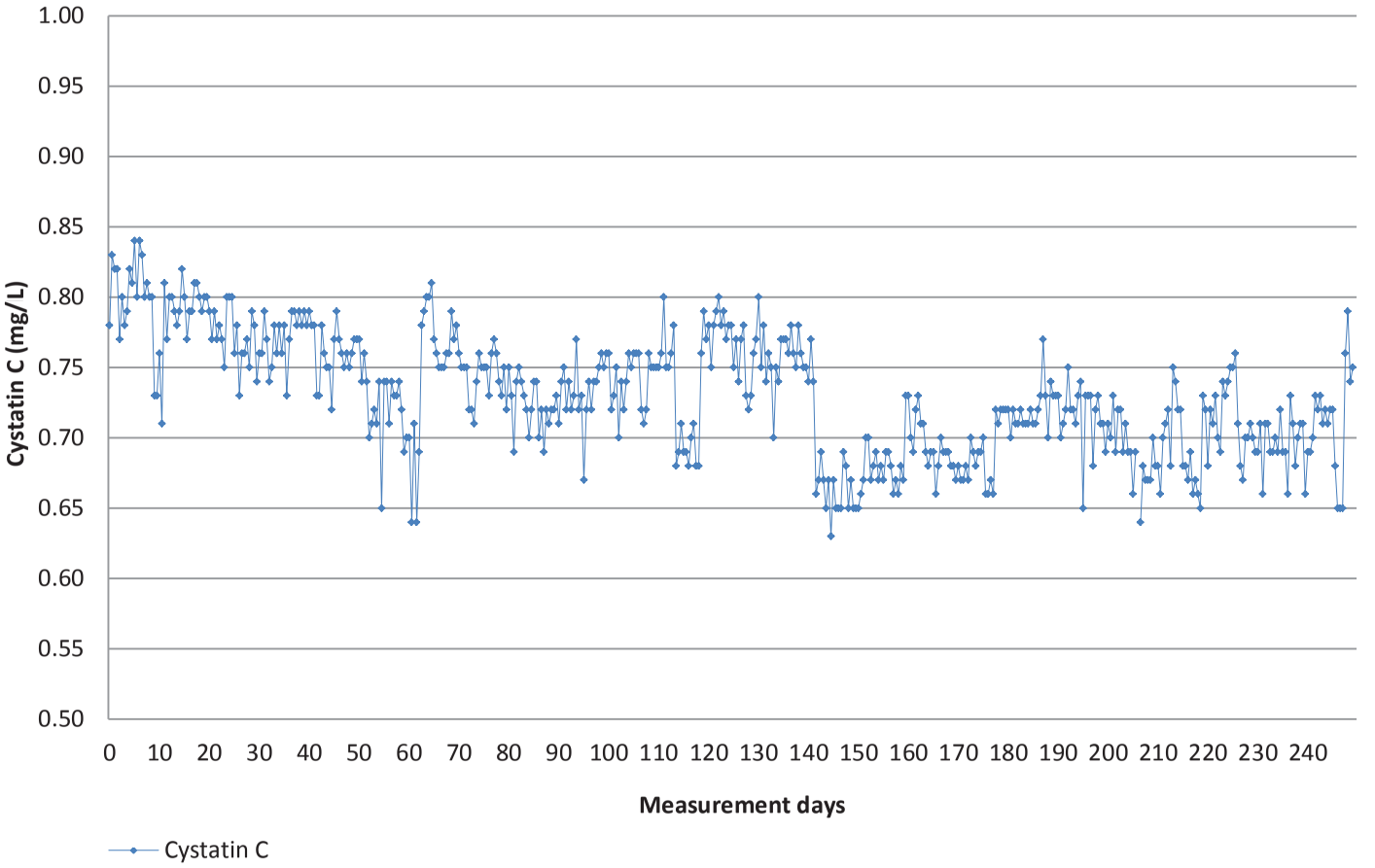

Table 1 | **Characteristics of PREVEND study participants overall and according to categories of $eGFR_{cysC\ corr}$**

		Categories of $eGFR_{cysC\ corr}$ (mL/min/1.73 m^2)					
	Overall (N=8,030)	≥120 (n=459)	90-119 (n=4,662)	75-89 (n=1,559)	60-74 (n=902)	45-59 (n=331)	<45 (n=77)
Age (years)	49.1 ± 12.6	36.4 ± 6.7	44.5 ± 9.9	54.5 ± 11.4	61.9 ± 9.5	66.5 ± 6.8	67.2 ± 7.9
Gender (male)	3,996 (50)	284 (62)	2,141 (46)	846 (53)	488 (54)	189 (57)	48 (62)
Race (Caucasians)	7,614 (96)	415 (91)	4,387 (95)	1,547 (97)	876 (98)	315 (96)	74 (96)
Current smoking (%)	3,020 (38)	132 (29)	1,677 (36)	704 (44)	357 (40)	125 (38)	25 (32)
Body Mass Index (kg/m^2)	26.1 ± 4.2	24.7 ± 3.5	25.5 ± 4.1	26.9 ± 4.3	27.5 ± 4.3	27.9 ± 4.3	27.3 ± 4.4
Blood glucose (mmol/L)	4.9 ± 1.2	4.6 ± 0.9	4.8 ± 1.2	5.0 ± 1.3	5.1 ± 1.1	5.3 ± 1.3	5.3 ± 1.1
Diabetes mellitus (%)	249 (3)	9 (2)	96 (2)	62 (4)	51 (6)	23 (7)	8 (11)
Total cholesterol (mmol/L)	5.6 ± 1.2	5.1 ± 1.0	5.5 ± 1.1	5.9 ± 1.1	6.0 ± 1.1	6.0 ± 1.2	5.9 ± 1.3
Hypercholesterolemia (%)	2,388 (29)	65 (14)	1,164 (25)	633 (40)	367 (41)	130 (40)	29 (38)
Systolic blood pressure (mm Hg)	128.8 ± 20.1	121.9 ± 14.6	124.7 ± 17.8	132.4 ± 20.0	139.9 ± 22.4	146.1 ± 24.3	145.8 ± 21.6
Diastolic blood pressure (mm Hg)	73.9 ± 9.7	69.8 ± 8.5	72.6 ± 9.4	75.8 ± 9.4	77.7 ± 9.6	78.9 ± 10.1	79.5 ± 10.5
Hypertension (%)	2,581 (33)	22 (5)	563 (12)	363 (23)	373 (41)	191 (58)	54 (70)
Urinary albumin (mg/24-h)	9.4 (6.3-17.8)	14.7 (6.6-13.1)	8.7 (6.1-14.7)	9.8 (6.4-20.0)	11.8 (6.9-29.1)	18.8 (8.4-59.2)	62.7 (18.8-288.6)
Albuminuria (≥30mg/24-h)(%)	1,208 (15)	39 (8.5)	512 (11)	262 (16)	220 (24)	122 (37)	53 (69)
Cystatin C (mg/L)(non-corrected)	0.90 ± 0.02	0.66 ± 0.08	0.82 ± 0.09	0.96 ± 0.07	1.08 ± 0.08	1.28 ± 0.12	1.87 ± 0.74
$eGFR_{cysC}$ (mL/min/1.73 m^2)	92.8 ± 19.3	123.3 ± 6.2	102.2 ± 10.2	82.0 ± 7.6	67.7 ± 6.7	53.8 ± 6.2	35.4 ± 8.4
Cystatin C (mg/L)(corrected)	0.88 ± 0.19	0.65 ± 0.07	0.80 ± 0.07	0.94 ± 0.05	1.07 ± 0.06	1.26 ± 0.09	1.83 ± 0.69
$eGFR_{cysC\ corr}$ (mL/min/1.73 m^2)	94.9 ± 19.0	124.4 ± 4.8	105.1 ± 8.2	83.1 ± 4.3	68.6 ± 4.2	54.1 ± 4.4	36.2 ± 8.5

Continuous variables are presented as means ± standard deviation or median (interquartile range) and categorical variables are presented as number (percentages)
Abbreviations are: $eGFR_{cysC\ corr}$=estimated Glomerular Filtration Rate from corrected cystatin C, $eGFR_{cysC}$ = estimated Glomerular Filtration Rate from routinely measured cystatin.

Association of $eGFR_{cysC\ corr}$ and $eGFR_{cysC}$ with CKD risk factors

Results of univariate and multivariate linear regression analyses are shown in Table 2. In general, associations of CKD risk factors were stronger with $eGFR_{cysC\ corr}$ than with $eGFR_{cysC..}$ In the multivariate linear regression model, we included age, sex, body mass index, total cholesterol, UAE, systolic blood pressure, glucose and smoking. The adjusted R-square of the multivariate linear regression model for $eGFR_{cysC\ corr}$ was higher than for $eGFR_{cysC}$ (0.479 vs. 0.458, p <0.001).

Table 2 | **Cross-sectional associations of $eGFR_{cysC}$ and $eGFR_{cysC\ corr}$ with chronic kidney disease risk factors** ***(Upper panel shows results of univariate analyses and lower panel results of the multivariate model)***

	eGFRcysC			eGFRcysC corr		
	beta	P-value	R-square	beta	P-value	R-square
Univariate linear regression						
Age (year)	-0.65	<0.001	0.419	-0.66	<0.001	0.443*
Sex (female)	0.06	<0.001	0.003	0.06	<0.001	0.003
Body Mass Index (kg/m^2)	-0.22	<0.001	0.049	-0.24	<0.001	0.056*
Total cholesterol (mmol/L)	-0.23	<0.001	0.053	-0.24	<0.001	0.055
UAE (mg/24h, log)	-0.16	<0.001	0.419	-0.16	<0.001	0.419
Systolic blood pressure (mmHg)	-0.34	<0.001	0.116	-0.35	<0.001	0.121*
Glucose (mmol/L)	-0.12	<0.001	0.015	-0.16	<0.001	0.026*
Smoking (current)	-0.08	<0.001	0.006	-0.07	<0.001	0.005
Multivariate linear regression						
Age (year)	-0.64	<0.001		-0.66	<0.001	
Sex (female)	-0.01	0.222		-0.02	0.12	
Body Mass Index (kg/m^2)	-0.07	<0.001		-0.08	<0.001	
Total cholesterol (mmol/L)	0.01	0.341	0.453	0.01	0.19	0.479*
UAE (mg/24h, log)	-0.09	<0.001		-0.10	<0.001	
Systolic blood pressure (mmHg)	-0.04	<0.001		-0.05	0.001	
Glucose (mmol/L)	0.08	<0.001		0.07	<0.001	
Smoking (current)	-0.15	<0.001		-0.16	<0.001	

Abbreviations are: $eGFR_{cys}$=GFR estimated from routinely measured cystatin C; $eGFR_{cys\ corr}$=GFR estimated from corrected cystatin C; UAE=Urinary Albumin Excretion
*p <0.05 (for difference in R-square)

Number and percentage of reclassified and non-reclassified participants

Of participants with an $eGFR_{cysC}$ of 90-119, 75-89, 60-74, 45-59 and <45 mL/min/1.73 m^2, 4% (n=140), 36% (n =664), 30% (n=310), 31% (n=118) and 25% (n=24) were reclassified upward to a higher $eGFR_{cysC\ corr}$ category, whereas of participants with an $eGFR_{cysC}$ of ≥120, 90-119, 75-89, 60-74, and 45-59 mL/min/1.73 m^2, 26% (n=114), 6% (n=258), 7% (n=126), 6% (n=54) and 2% (n=6) were reclassified downward to a lower $eGFR_{cysC\ corr}$ category (Table 3).

Table 3 | **Number and percentage of participants reclassified up- and downward and non-reclassified participants using eGFR derived corrected cystatin C instead of routinely measured cystatin C**

$eGFR_{cys}$ category (ml/min/1.73 m^2)	Participants in each category N	Upward reclassification n (%)	No reclassification n (%)	Downward reclassification n (%)
≥120	433	NA	319 (74)	114 (26)
90 - 119	4,282	140 (4)	3,884 (90)	258 (6)
75 - 89	1,821	664 (36)	1,031 (57)	126 (7)
60 - 74	1,022	310 (30)	658 (64)	54 (6)
45 - 59	377	118 (31)	253 (67)	6 (2)
<45	95	24 (25)	71 (75)	NA

Abbreviations are: $eGFR_{cys}$= GFR estimated from routinely measured cystatin C, NA=not applicable

Means and proportion of CKD risk factors in reclassified and non-reclassified participants

Participants who were reclassified upward to a higher eGFR category were in general younger and had lower systolic blood pressure, body mass index, total cholesterol, blood glucose and UAE than those who were not reclassified, i.e. who stayed in the same eGFR category (Table 4). The opposite was true for participants who were reclassified downward. Compared to non-reclassified participants, the proportion of current smokers was lower in participants who were reclassified upward to a higher eGFR category and higher in participants who were reclassified downward to a lower eGFR category (Table 4).

Table 4 | **Clinical characteristics of reclassified and non-reclassified participants**

$eGFR_{cys}$ category (ml/min/1.73 m^2)	Upward reclassification	No reclassification	Downward reclassification	p
Age (years)				
≥120	NA	36.5 ± 7.0	37.4 ± 5.8	0.26
90 - 119	36.0 ± 5.8	43.9 ± 9.6	49.9 ± 10.9	<0.001
75 - 89	49.0 ± 10.8	54.6 ± 11.3	57.5 ± 10.7	<0.001
60 - 74	58.2 ± 10.4	62.2 ± 9.3	63.6 ± 7.8	<0.001
45 - 59	65.2 ± 7.4	67.1 ± 6.4	70.5 ± 5.3	0.01
<45	65.7 ± 6.9	66.9 ± 8.1	NA	0.51
Systolic blood pressure (mmHg)				
≥120	NA	122.1 ± 14.9	119.9 ± 12.9	0.17
90 - 119	121.6 ± 13.8	124.2 ± 17.6	128.8 ± 17.1	<0.001
75 - 89	128.4 ± 19.1	132.4 ± 20.4	136.0 ± 22.4	<0.001
60 - 74	135.4 ± 20.5	139.7 ± 21.9	137.6 ± 23.2	0.02
45 - 59	144.9 ± 24.6	147.3 ± 23.7	147.3 ± 26.5	0.67
<45	153.0 ± 28.7	145.7 ± 22.1	NA	0.20
Total cholesterol (mmol/L)				
≥120	NA	5.0 ± 0.9	5.1 ± 0.8	0.57
90 - 119	5.2 ± 0.9	5.5 ± 1.1	5.7 ± 1.1	<0.001
75 - 89	5.7 ± 1.1	5.9 ± 1.1	6.0 ± 1.1	<0.001
60 - 74	5.9 ± 1.1	5.9 ± 1.1	6.1 ± 1.5	0.73
45 - 59	5.9 ± 1.1	5.9 ± 1.1	6.1 ± 1.2	0.9
<45	6.1 ± 1.4	5.9 ± 1.3	NA	0.68
Blood glucose (mmol/L)				
≥120	NA	4.7 ± 0.9	4.8 ± 0.7	0.28
90 - 119	4.4 ± 0.7	4.8 ± 1.1	5.1 ± 0.9	<0.001
75 - 89	4.7 ± 1.3	5.0 ± 1.4	5.1 ± 0.8	<0.001
60 - 74	4.9 ± 1.3	5.1 ± 1.1	5.4 ± 1.2	0.004
45 - 59	5.1 ± 1.4	5.3 ± 1.3	5.0 ± 0.5	0.30
<45	4.8 ± 0.7	5.4 ± 1.2	NA	0.03
Body Mass Index (kg/m^2)				
≥120	NA	24.6 ± 3.4	24.8 ± 4.3	0.66
90 - 119	24.8 ± 3.5	25.4 ± 3.9	26.6 ± 4.0	<0.001
75 - 89	26.3 ± 4.4	26.9 ± 4.4	27.2 ± 4.4	0.003
60 - 74	27.2 ± 4.2	27.4 ± 4.2	27.6 ± 4.8	0.57
45 - 59	28.0 ± 4.2	28.0 ± 4.2	25.9 ± 6.1	0.47
<45	28.3 ± 3.2	27.4 ± 4.6	NA	0.39
Albuminuria (mg/24h, log)				
≥120	NA	8.8 (6.6 – 14.4)	9.2 (6.7 – 13.8)	0.76
90 - 119	8.6 (6.7 – 11.4)	8.7 (6.1 – 14.7)	9.3 (6.2 – 17.1)	0.59
75 - 89	8.3 (5.9 – 14.8)	9.7 (6.4 – 21.3)	10.5 (6.7 – 26.0)	<0.001
60 - 74	10.5 (6.4 – 20.7)	11.9 (6.8 – 28.1)	9.7 (5.5 – 22.8)	0.04
45 - 59	15.5 (7.8 – 53.1)	19.3 (9.2 – 59.5)	22.5 (5.6 – 36.7)	0.46
<45	41.2 (16.1 – 98.6)	75.9 (19.2 – 326.8)	NA	0.13
Smoking (current)				
≥120	NA	28	23	0.30
90 - 119	31	35	45	0.004
75 - 89	43	43	43	0.9
60 - 74	45	39	47	0.09
45 - 59	37	40	33	0.91
<45	30	32	NA	0.86

Abbreviations are: $eGFR_{cys}$= GFR estimated from routinely measured cystatin C, NA=not applicable

Incident cardiovascular events in reclassified participants

A total of 789 participants experienced a CV event during follow-up of 9.3 ± 2.7 years. Compared to non-reclassified participants, the crude incidence rate per 1000 person years tended to be higher in participants classified downward to a lower eGFR category and lower in participants classified to upward to a higher eGFR category using the $eGFR_{cysC\ corr}$. Downward reclassification was associated with significantly higher risk of incident CV events and upward reclassification with significantly lower risk (Table 5).

Table 5 | **Cardiovascular morbidity and mortality according to reclassification status by $eGFR_{cys\ corr}$ (ml/min/1.73 m^2) compared with $eGFR_{cys}$ (ml/min/1.73 m^2)**

	By $eGFR_{cys\ corr}$ (ml/min/1.73 m^2) category reclassification		
$eGFR_{cys}$ category (ml/min/1.73 m^2)	Upward	None	Downward
≥120			
Participants reclassified (N)	NA	319	114
Events reclassified (n)	NA	11	0
Crude incidence rate (1000 PY)	NA	3.3	NR
Hazard ratio	NA	Reference	NR
90-119			
Participants reclassified (N)	140	3,884	258
Events reclassified (n)	1	182	25
Crude incidence rate (1000 PY)	NR	4.6	8.9
Hazard ratio	NR	Reference	1.93 (1.22 – 3.03)
75-89			
Participants reclassified (N)	664	1,031	126
Events reclassified (n)	62	128	21
Crude incidence rate (1000 PY)	10.2	13.6	17.6
Hazard ratio	0.74 (0.55 – 0.99)	Reference	1.63 (1.07 – 2.11)
60-74			
Participants reclassified (N)	310	658	54
Events reclassified (n)	49	143	10
Crude incidence rate (1000 PY)	17.2	24.7	23.3
Hazard ratio	0.68 (0.49 – 0.94)	Reference	1.08 (0.65 – 1.54)
45-59			
Participants reclassified (N)	118	253	6
Events reclassified (n)	36	88	1
Crude incidence rate (1000 PY)	39.5	46.5	NR
Hazard ratio	0.72 (0.50 – 0.98)	Reference	NR
<45			
Participants reclassified (N)	24	71	NA
Events reclassified (n)	8	24	NA
Crude incidence rate (1000 PY)	46.8	51.3	NA
Hazard ratio	0.79 (0.56 – 1.00)	Reference	NA

Abbreviations are: $eGFR_{cys}$= GFR estimated from routinely measured cystatin C; $eGFR_{cys\ corr}$= GFR estimated from corrected cystatin C; PY = person years; NA = not available; NR = not reliable

Net reclassification improvement

An NRI analysis was conducted for incident CV events based on eGFR categories for upward as well as downward reclassified participants with reliable numbers of incident CV events (i.e. n ≥5). A total of 180 participants were reclassified to higher risk category using the $eGFR_{cysC\ corr}$ (i.e. a lower eGFR), of which 31 (17%) participants experienced a CV event during follow-up. In contrast, 998 participants were reclassified to a lower risk (i.e. higher eGFR) using the $eGFR_{cysC\ corr}$, of which 119 (12%) had an incident CV event. NRI for incident CV events using the $eGFR_{cysC\ corr}$ was positive and significant, i.e. 0.102 (p=0.019).

Replication study

A brief description of the ESTHER study is provided in Appendix Text. Characteristics of ESTHER study participants are shown in Appendix Table 1. Drift and day-to-day variability in cystatin C measurements can be seen in Appendix Figure 1. For this study reference material was available for the lower and higher cystatin C concentration range (left and right panel, respectively). A strong correlation was observed between both parameters that nearly equaled the line of identity (β=0.88 and p <0.001, Appendix Figures 2 and 3).

In the ESTHER study, results from univariate and multivariate regression analyses of $eGFR_{cysC\ corr}$ or $eGFR_{cysC}$ with CKD risk factors, and redistribution of clinical characteristics in reclassified participants were essentially similar to the results obtained in the PREVEND study (Appendix Tables 2-4). Although the adjusted R-square of the multivariate linear regression model in ESTHER was lower than in PREVEND, which may be explained by the narrower age range of the ESTHER cohort, it was again higher for $eGFR_{cysC\ corr}$ than for $eGFR_{cysC}$ (0.231 vs. 0.186) (Appendix Table 2) and the difference was statistically significant (p <0.001). Compared to non-reclassified participants, risk of incident CV events was higher in participants reclassified downwards while risk was lower in participants reclassified upwards (Appendix Table 5). In this study, NRI using the $eGFR_{cysC\ corr}$ was also statistically significant for incident CV events (0.019; p=0.046).

DISCUSSION

Our results show that use of a day-to-day specific correction factor to adjust for drift and day-to-day variation in cystatin C measurement results in an $eGFR_{cysC\ corr}$ that has stronger associations with established CKD risk factors than $eGFR_{cysC}$. Categorization of kidney function using the $eGFR_{cysC\ corr}$ more appropriately stratifies individuals according to their CKD risk factors compared with the $eGFR_{cysC}$. Importantly, reclassification using $eGFR_{cysC\ corr}$ instead of $eGFR_{cysC}$ led to better prediction of incident CV morbidity and mortality.

Previous studies reported day-to-day variation in measurement of serum and urinary creatinine (18), and spot urine albumin-creatinine ratio (19). These studies suggested considering such variability in the measurement of CKD markers while classifying individuals in CKD stages. Another study showed day-to-day variation in cystatin C occurring from biological reasons (20). However, this is the first study to investigate the importance of correcting drift and day-to-day variation in cystatin C measurements that can occur during measurement despite standard laboratory quality control.

Variability in measurements of cystatin C concentration may occur because of a number of reasons. It can be due to random variation, and due to true short-term variation and drift that occurs slowly over time when using analytical instruments. As seen in Figure 1, the measured cystatin C concentrations showed a tendency to decrease over several days and to increase sharply periodically. Adjustment for random variation will not result in an improvement in the association of $eGFR_{cysC}$ with CKD risk factors and CV risk that we observed. However, adjustment for true short-term variation and for drift occurring slowly over time using our day-specific correction factor is expected to do so. Because this procedure corrects for measurement error it will provide an estimate of cystatin C concentration that is closer to its true value. In the ESTHER study reference material was available for the lower as well as the higher cystatin C concentration range. Variation in both parameters were highly correlated, indicating that variability in cystatin C measurement was indeed not random variation, but true short-term variation and drift that occurs slowly over time (Appendix Figure 2 and Appendix Figure 3). Of note, although we did not investigate this in the present study, we think that it is unlikely that day-to-day variation and drift is specific for cystatin C measurement, but that this will also be observed for other analytes. What the value will be of the present correction method to improve associations with creatinine based eGFR values with CKD risk factors and prognosis needs to be studied separately.

Concentration of corrected cystatin C was lower than of the routinely measured cystatin C in the PREVEND study, whereas opposite was true in the ESTHER study. As a result, $eGFR_{cysC\ corr}$ was higher than $eGFR_{cysC}$ in the PREVEND study whereas the opposite was true in the ESTHER study. This might be because PREVEND and ESTHER used different assays, because measurements in the two studies were performed in different time periods, or because day-to-day variation and drift in the specific period that measurements took place in a study by chance resulted in for instance an overall higher $eGFR_{cysC\ corr}$, whereas for that same study in another time period the reverse could have been true.

Differences in clinical characteristics of reclassified participants after correction for day-to-day variation in cystatin C might have occurred because subjects that are reclassified upward are probably already in the higher range of their original eGFR category, and subjects that are reclassified downward are in the lower part. Since within each eGFR category eGFR is negatively

associated with age this may explain that subjects that are reclassified upward are younger compared to non-reclassified subjects, and vice versa, those subjects that are reclassified downward are older. A similar line of reasoning is expected to hold true for other CKD risk factors. Moreover, similar age and gender adjusted cardiovascular event rates (data not shown) in the reclassified participants and in the groups of participants they were reclassified to indicate that this indeed might be the case.

Our study has a number of strengths. First, with inclusion of plasma pool samples at each measurement day we were able to identify drift and day-to-day variation in cystatin C measurement. Second, cystatin C was measured under standard quality control from a large number of participants who also had information available on a large number of relevant covariates. Third, to confirm the findings obtained in the PREVEND cohort, we performed similar analyses in a replication study. The results obtained in the ESTHER cohort corroborate our primary results. Consequently, essentially similar results were obtained in two independent cohorts that made use of two different laboratories, both using standard laboratory quality control, and using two different cystatin C assays. This makes it likely that our results are generalizable and not dependent on laboratory or assay specific characteristics.

This study has also limitations, the most important being that in absence of a gold standard measurement of GFR, it is not possible to conclude definitively whether correction for drift and day-to-day variability in cystatin C measurement improves accuracy of GFR estimation. It should be noted that a gold standard measurement of GFR, such as with inulin or iothalamate, is not feasible in large scale observational studies, such as the PREVEND and ESTHER studies. Improvement in accuracy of estimation of true GFR might also be determined when comparing $eGFR_{cysC}$ and $eGFR_{cysC\ corr}$ with eGFR obtained with the CKD-EPI 2012 equation that uses creatinine as well as cystatin C (6). The likelihood of day-to-day variability and drift also in creatinine measurements, however, might dilute the effect of the specific cystatin C correction in estimation of GFR. Unfortunately, we did not have information on creatinine correction in our studies and therefore did not make above comparison. However, our results with respect to improvement in associations of eGFR with CKD risk factors and eGFR based prediction of prognosis, strongly suggest that correction in drift and day-today variation in cystatin C measurements results in improved accuracy of estimation of true GFR. Moreover, the present study did not investigate the causes for drift and day-to-day variability in cystatin C measurement. Answering this question is beyond the scope of the present study and requires additional investigation.

The results of our study may have important implications. In clinical practice, awareness of drift and day-to-day variability in cystatin C measurement (and consequently GFR estimation) may aid to prevent false diagnoses of CKD and, therefore, reiterate the importance of a second measurement of eGFR to confirm the diagnosis of CKD. For future epidemiological and clinical

research, correction for drift and day-to-day variability in cystatin C measurement can help in achieving research objectives with smaller studies and smaller randomized clinical trials, because it is to be expected to result in less variability in GFR estimates. In addition, compared to the other efforts made to improve accuracy of GFR estimation, such as the development of new estimation equations or new filtration markers, correction for drift and day-to-day variability in measurement of presently accepted filtration markers can prove a less cumbersome step. Thus, our study makes an important contribution by providing scientific evidence on the relevance of correction for drift and day-to-day variability in cystatin C measurement and GFR estimation.

In conclusion, our results, obtained in two independent cohorts both using standard quality control but using two different assays for cystatin C, show that correction for drift and day-to-day variation in cystatin C measurement improves associations of cystatin C derived eGFR with CKD risk factors and eGFR based risk classification for incident CV events.

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APPENDIX

The ESTHER study (N=9,949): Study description

ESTHER [„Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung“ (in German)] is an ongoing cohort study, details of which have been published elsewhere (1). In summary, 9949 men and women, aged 50-75 years at baseline, were recruited. Recruitment was performed by their general practitioners during a routine health check-up between 2000 and 2002 in the German federal state of Saarland.

For the current analysis participants with missing data on cystatin C and/or missing data on the date of measurement of reference samples (n=443) were excluded, leaving a total of 9,506 participants. The ESTHER study has been approved by the ethics committee of the University of Heidelberg and the medical board of the State of Saarland.

Measurements

Blood samples were obtained from all participants. Using the cystatin C based CKD-EPI equation (2), GFR was estimated from routinely measured cystatin C concentration (i.e. $eGFR_{cysC}$) and also from cystatin C corrected for day-to-day variation in measurement (i.e. $eGFR_{cysC\ corr}$).

Total cholesterol measurements were determined from serum samples by a high-performance liquid chromatography method calibrated with the Synchron LX multicalibrator system (Beckman Coulter, Galway, Ireland). All measurements were performed in a blinded fashion. Urinary albumin concentration was measured with an immunonephelometry assay (interassay CV 5.2%) in spot urine sample. Plasma glucose was assessed and documented on a standardised form by the general practitioners during the health check-up. The urinary albumin excretion (UAE) was calculated as albumin to creatinine ratio.

Information on age, sex, race and smoking were obtained by a standardized questionnaire. Current smoking was defined as current daily smoking of any tobacco products. Information on blood pressure, height, and weight were assessed by the general practitioners during the health check-up.

Incident cardiovascular events

We defined incident CV events as incidence of myocardial infarction, stroke or cardiovascular death. Vital status and date of deaths were ascertained through record linkage with the Saarland population registries. Cardiovascular deaths were identified via death certificates obtained from public health departments. All deaths coded with ICD-10 code I were counted. The ICD code for the leading cause of death from death certificates was available for 92.4% of the deceased.

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Appendix Table 1 | **Characteristics of ESTHER study participants overall and according to categories of $eGFR_{cysC\ corr}$**

		Categories of eGFR (mL/min/1.73 m^2)					
Characteristics	Overall* (N=9,506)	≥120 (n=174)	90-119 (n=3,328)	75-89 (n=2,829)	60-74 (n=2,071)	45-59 (n=862)	<45 (n=241)
Age (years)	62.1 ± 6.6	61.9 ± 7.1	59.1 ± 6.1	61.8 ± 6.2	64.8 ± 5.8	66.7 ± 5.7	67.7 ± 7.4
Gender (male)	4,203 (44)	100 (57)	1,560 (47)	1,224 (43)	879 (42)	342 (40)	98 (40)
Race (Caucasians)	9,432 (99)	168 (97)	3,304 (99)	2,807 (99)	2,054 (99)	858 (99)	241 (99)
Current smoking (%)	1,561 (17)	30 (18)	540 (17)	485 (18)	330 (16)	135 (16)	41 (18)
Body Mass Index (kg/m^2)	27.7 ± 4.4	27.5 ± 4.1	26.9 ± 4.2	27.7 ± 4.2	28.3 ± 4.4	28.7 ± 4.9	29.0 ± 5.4
Blood glucose (mmol/L)	5.6 ± 1.9	5.5 ± 1.7	5.5 ± 1.9	5.6 ± 1.7	5.6 ± 1.7	5.9 ± 2.1	6.5 ± 2.9
Diabetes mellitus (%)	1,362 (15)	24 (15)	412 (13)	359 (13)	296 (15)	187 (23)	84 (36)
Total serum cholesterol (mmol/L)	5.7 ± 1.3	5.6 ± 1.3	5.6 ± 1.4	5.7 ± 1.3	5.7 ± 1.3	5.8 ± 1.3	5.5 ± 1.3
Hypercholesterolemia (%)	3,226 (34)	57 (33)	1,073 (32)	994 (35)	727 (35)	310 (36)	67 (28)
Systolic blood pressure (mm Hg)	139.9 ± 19.6	138.7 ± 18.4	137.4 ± 19.3	140.1 ± 19.6	142.2 ± 19.4	142.3 ± 19.7	145.4 ± 20.1
Diastolic blood pressure (mm Hg)	83.7 ± 10.2	83.7 ± 10.2	83.3 ± 10.2	83.9 ± 10.2	84.3 ± 10.4	83.4 ± 9.8	83.0 ± 10.0
Hypertension (%)	7,007 (74)	125 (72)	2,196 (70)	2,069 (73)	1,651 (80)	737 (85)	229 (95)
Albumin-Creatinine ratio (mg/g)	9.5 (5.9-18.5)	7.9 (4.8-18.0)	8.9 (5.9-16.7)	8.9 (5.7-16.5)	9.6 (6.1-18.4)	12.7 (7.1-28.5)	28.7 (11.2-253.4)
Albuminuria (≥30mg/24-hr) (%)	1,226 (16)	23 (16)	368 (13)	291 (12)	260 (15)	177 (24)	107 (48)
Cystatin C (mg/L) (non-corrected)	0.93 ± 0.27	0.49 ± 0.17	0.79 ± 0.08	0.91 ± 0.06	1.04 ± 0.08	1.22 ± 0.10	1.82 ± 0.90
$eGFR_{cysC}$ (mL/min/1.73 m^2)	83.6 ± 20.5	156.0 ± 109.7	100.1 ± 7.9	84.6 ± 7.1	70.5 ± 6.4	55.9 ± 5.5	36.9 ± 9.1
Cystatin C (mg/L) (corrected)	0.95 ± 0.28	0.47 ± 0.14	0.78 ± 0.07	0.92 ± 0.04	1.06 ± 0.06	1.25 ± 0.08	1.85 ± 0.90
$eGFR_{cysC\ corr}$ (mL/min/1.73 m^2)	82.3 ± 19.1	143.6 ±55.7	100.5 ± 6.7	82.5 ± 4.3	68.2 ± 4.3	54.0 ± 4.0	35.8 ± 8.4

Continuous variables are presented as means ± standard deviation or median (interquartile range) and categorical variables are presented as number (percentages).
Abbreviations are: ESTHER ["Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung" (in German)] , $eGFR_{cysC\ corr}$=estimated Glomerular filtration rate from corrected cystatin C, $eGFR_{cysC}$ =estimated glomerular filtration rate from routinely measured cystatin C. Note: 443 participants were missing information on cystatin C

Appendix Table 2 | **Cross-sectional associations of renal risk factors with $eGFR_{cysC}$ and $eGFR_{cysC\ corr}$**
(Upper panel shows results of univariate analyses and lower panel results of the multivariate model)

	$eGFR_{cysC}$			$eGFR_{cysC\ corr}$		
Factors	**beta**	**P-value**	**R-square**	**beta**	**P-value**	**R-square**
Univariate linear regression						
Age (year)	-0.38	<0.001	0.146	-0.42	<0.001	0.176*
Sex (female)	-0.07	<0.001	0.005	-0.07	<0.001	0.005
Body Mass Index (kg/m^2)	-0.14	<0.001	0.020	-0.15	<0.001	0.022*
Total cholesterol (mmol/L)	0.02	0.113	0.000	-0.03	0.004	0.001
UAE (mg/g, log)	-0.13	<0.001	0.017	-0.14	<0.001	0.020*
Systolic blood pressure (mmHg)	-0.09	<0.001	0.009	-0.11	<0.001	0.013*
Glucose (mmol/L)	-0.05	<0.001	0.003	-0.06	<0.001	0.004
Smoking (current)	0.01	0.263	0.000	0.01	0.185	0.000
Multivariate linear regression						
Age (year)	-0.38	<0.001		-0.43	<0.001	
Sex (female)	-0.10	<0.001		-0.10	<0.001	
Body Mass Index (kg/m^2)	-0.12	<0.001		-0.13	<0.001	
Total cholesterol (mmol/L)	0.03	0.001	0.186	-0.01	0.250	0.231*
UAE (mg/g, log)	-0.11	<0.001		-0.12	<0.001	
Systolic blood pressure (mmHg)	0.01	0.852		-0.01	0.988	
Glucose (mmol/L)	0.03	0.004		0.04	<0.001	
Smoking (current)	-0.09	<0.001		-0.09	<0.001	

Abbreviations are: $eGFR_{cys}$=GFR estimated from routinely measured cystatin C, $eGFR_{cys\ corr}$=GFR estimated from corrected cystatin C, UAE=Urinary Albumin Excretion
*p <0.05 (for difference in R-square)

Appendix Table 3 | **Number and percentage reclassified and non-reclassified participants after estimating GFR from corrected cystatin C**

$eGFR_{cys}$ category ($ml/min/1.73\ m^2$)	Participants in each category (N=9,506)	Upward reclassification	No reclassification	Downward reclassification
≥120	166	NA	156 (94)	10 (6)
90 - 119	3,632	18 (0.5)	2,959 (81)	654 (18)
75 - 89	2,778	359 (13)	1,901 (68)	518 (19)
60 - 74	1,950	274 (14)	1,457 (75)	219 (11)
45 - 59	756	95 (13)	622 (82)	39 (5)
<45	224	21 (9)	203 (91)	NA

Abbreviation is: $eGFR_{cys}$=GFR estimated from routinely measured cystatin C using the CKD-EPI equation (cystatin C only)

Appendix Table 4 | **Clinical characteristics of reclassified and non-reclassified participants**

$eGFR_{cys}$ category (ml/min/1.73 m^2)	Upward reclassification	No reclassification	Downward reclassification	p
Age (years)				
≥120	NA	62.8 ± 6.8	57.4 ± 11.9	0.003
90 - 119	54.1 ± 9.2	58.8 ± 6.0	60.6 ± 6.2	<0.001
75 - 89	61.4 ± 6.0	62.1 ± 6.3	63.3 ± 5.8	<0.001
60 - 74	63.9 ± 5.6	65.2 ± 5.6	66.1 ± 6.0	<0.001
45 - 59	65.5 ± 5.7	66.9 ± 5.5	67.4 ± 5.7	0.641
<45	66.7 ± 6.9	67.8 ± 5.4	NA	0.512
Systolic blood pressure (mmHg)				
≥120	NA	138.9 ± 18.3	134.7 ± 21.9	0.482
90 - 119	137.1 ± 20.3	137.2 ± 19.3	139.9 ± 19.8	0.012
75 - 89	139.7 ± 18.8	140.1 ± 19.6	141.3 ± 19.9	0.413
60 - 74	141.0 ± 18.7	142.7 ± 19.2	143.6 ± 20.8	0.243
45 - 59	141.1 ± 19.3	142.9 ± 19.3	147.0 ± 17.7	0.035
<45	137.8 ± 21.6	145.1 ± 20.5	NA	0.013
Total cholesterol (mmol/L)				
≥120	NA	5.6 ± 1.4	5.8 ± 0.6	0.873
90 - 119	5.6 ± 1.2	5.6 ± 1.3	6.0 ± 1.2	<0.001
75 - 89	5.3 ± 1.5	5.7 ± 1.3	6.0 ± 1.1	<0.001
60 - 74	5.2 ± 1.5	5.6 ± 1.3	6.0 ± 1.2	<0.001
45 - 59	5.3 ± 1.5	5.7 ± 1.4	5.9 ± 1.1	0.08
<45	5.5 ± 1.5	5.5 ± 1.4	NA	0.854
Blood glucose (mmol/L)				
≥120	NA	5.3 ± 1.3	5.8 ± 1.7	0.033
90 - 119	6.7 ± 3.5	5.4 ± 1.9	5.6 ± 1.2	0.013
75 - 89	5.4 ± 1.5	5.6 ± 1.8	5.8 ± 1.7	0.337
60 - 74	5.6 ± 1.7	5.7 ± 1.4	5.9 ± 2.2	0.017
45 - 59	5.5 ± 2.0	5.9 ± 2.1	6.0 ± 1.8	0.160
<45	6.6 ± 3.9	6.6 ± 3.1	NA	0.885
Body Mass Index (kg/m^2)				
≥120	NA	27.4 ± 4.0	27.4 ± 5.1	0.974
90 - 119	26.8 ± 4.2	27.3 ± 3.9	28.2 ± 5.3	0.015
75 - 89	27.7 ± 4.1	27.8 ± 4.2	28.2 ± 4.4	0.177
60 - 74	28.2 ± 4.5	28.4 ± 4.4	28.5 ± 4.8	0.567
45 - 59	28.5 ± 4.2	28.7 ± 4.8	29.6 ± 4.9	0.047
<45	30.2 ± 6.6	29.9 ± 5.5	NA	0.292
Albuminuria Median (mg/g)				
≥120	NA	7.8 (5.0 – 18.0)	11.9 (6.7 – 17.8)	0.009
90 - 119	9.1 (5.6 – 16.4)	8.9 (5.9 – 16.2)	10.4 (4.5 – 17.8)	0.927
75 - 89	9.5 (5.9 – 19.5)	8.7 (5.6 – 16.5)	8.9 (5.9 – 187)	0.106
60 - 74	9.9 (6.1 – 18.6)	10.8 (6.2 – 21.9)	9.6 (6.0 – 18.5)	0.046
45 - 59	9.8 (6.6 – 25.2)	13.8 (7.6 – 32.2)	14.1 (7.8 – 79.7)	0.035
<45	22.4 (8.6 – 45.9)	34.6 (12.5 – 324.4)	NA	0.206
Smoking (current)				
≥120	NA	18	20	0.304
90 - 119	11	16	16	0.893
75 - 89	18	19	14	0.824
60 - 74	14	17	17	0.148
45 - 59	15	18	23	0.049
<45	17	19	NA	0.790

Abbreviation is: $eGFR_{cys}$=GFR estimated from routinely measured cystatin C using the CKD-EPI equation (cystatin C only)

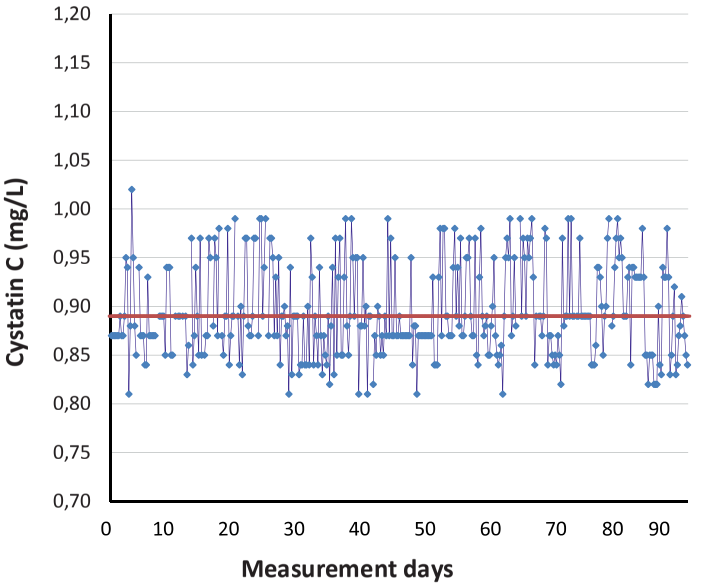
Appendix Table 5 | **Cardiovascular morbidity and mortality according to reclassification status by $eGFR_{cys\ corr}$ (ml/min/1.73 m^2) compared with $eGFR_{cys}$ (ml/min/1.73 m^2)**

	By $eGFR_{cys\ corr}$ (ml/min/1.73 m^2) category reclassification		
$eGFR_{cys}$ category (ml/min/1.73 m^2)	Upward (n=88)	None (n=908)	Downward (n=180)
≥120			
Participants reclassified (n)	NA	20	4
Crude incidence rate/1000 PY	NA	13.5	NR
Crude hazard ratio	NA	Reference	NR
Adjusted hazard ratio[a]	NA	Reference	NR
Adjusted hazard ratio[b]	NA	Reference	NR
90-119			
Participants reclassified (n)	3	283	64
Crude incidence rate/1000 PY	NR	10.3	10.6
Crude hazard ratio	NR	Reference	1.28 (1.02 – 1.61)
Adjusted hazard ratio[a]	NR	Reference	1.10 (0.84 – 1.46)
Adjusted hazard ratio[b]	NR	Reference	2.05 (0.66 – 6.43)
75-89			
Participants reclassified (n)	39	202	65
Crude incidence rate/1000 PY	11.6	11.6	13.8
Crude hazard ratio	0.81 (0.57 – 1.14)	Reference	1.42 (1.07 – 1.89)
Adjusted hazard ratio[a]	0.80 (0.57 – 1.13)	Reference	1.30 (0.98 – 1.73)
Adjusted hazard ratio[b]	0.99 (0.70 – 1.47)	Reference	1.19 (0.88 – 1.21)
60-74			
Participants reclassified (n)	27	223	38
Crude incidence rate/1000 PY	10.7	16.9	19.8
Crude hazard ratio	0.52 (0.34 – 0.76)	Reference	1.63 (1.15 – 2.30)
Adjusted hazard ratio[a]	0.53 (0.35 – 0.80)	Reference	1.58 (1.12 – 2.24)
Adjusted hazard ratio[b]	0.67 (0.43 – 1.03)	Reference	1.20 (0.82 – 1.75)
45-59			
Participants reclassified (n)	13	128	9
Crude incidence rate/1000 PY	15.9	24.2	30.3
Crude hazard ratio	0.59 (0.33 – 1.03)	Reference	1.35 (0.69 – 2.66)
Adjusted hazard ratio[a]	0.58 (0.32 – 1.01)	Reference	1.27 (0.65 – 2.51)
Adjusted hazard ratio[b]	0.85 (0.56 – 1.28)	Reference	0.77 (0.28 – 2.11)
<45			
Participants reclassified (n)	6	52	NA
Crude incidence rate/1000 PY	35.0	33.0	NA
Crude hazard ratio	0.95 (0.41 – 2.22)	Reference	NA
Adjusted hazard ratio[a]	0.97 (0.42 – 2.27)	Reference	NA
Adjusted hazard ratio[b]	0.80 (0.28 – 2.29)	Reference	NA

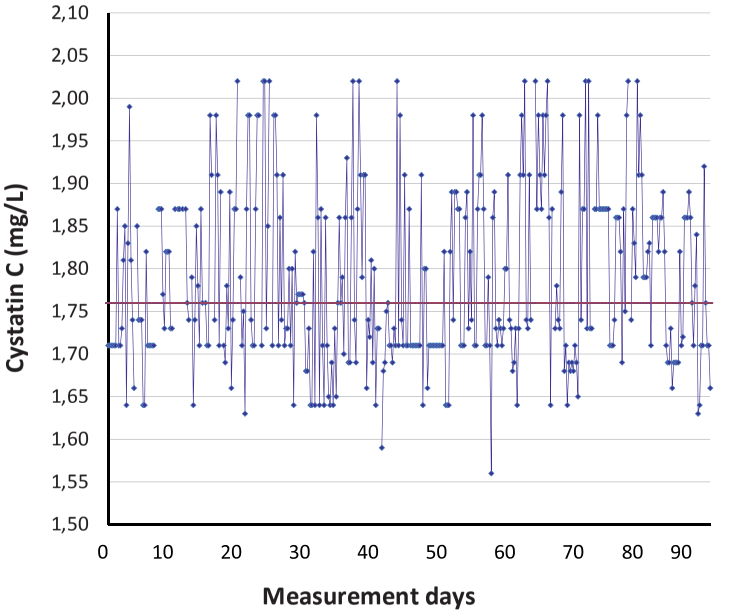
a=Adjusted for age and gender, b=a + BMI, hypertension, diabetes and CVD history
Abbreviations are: PY=person years, NA=not applicable, NR=not reliable

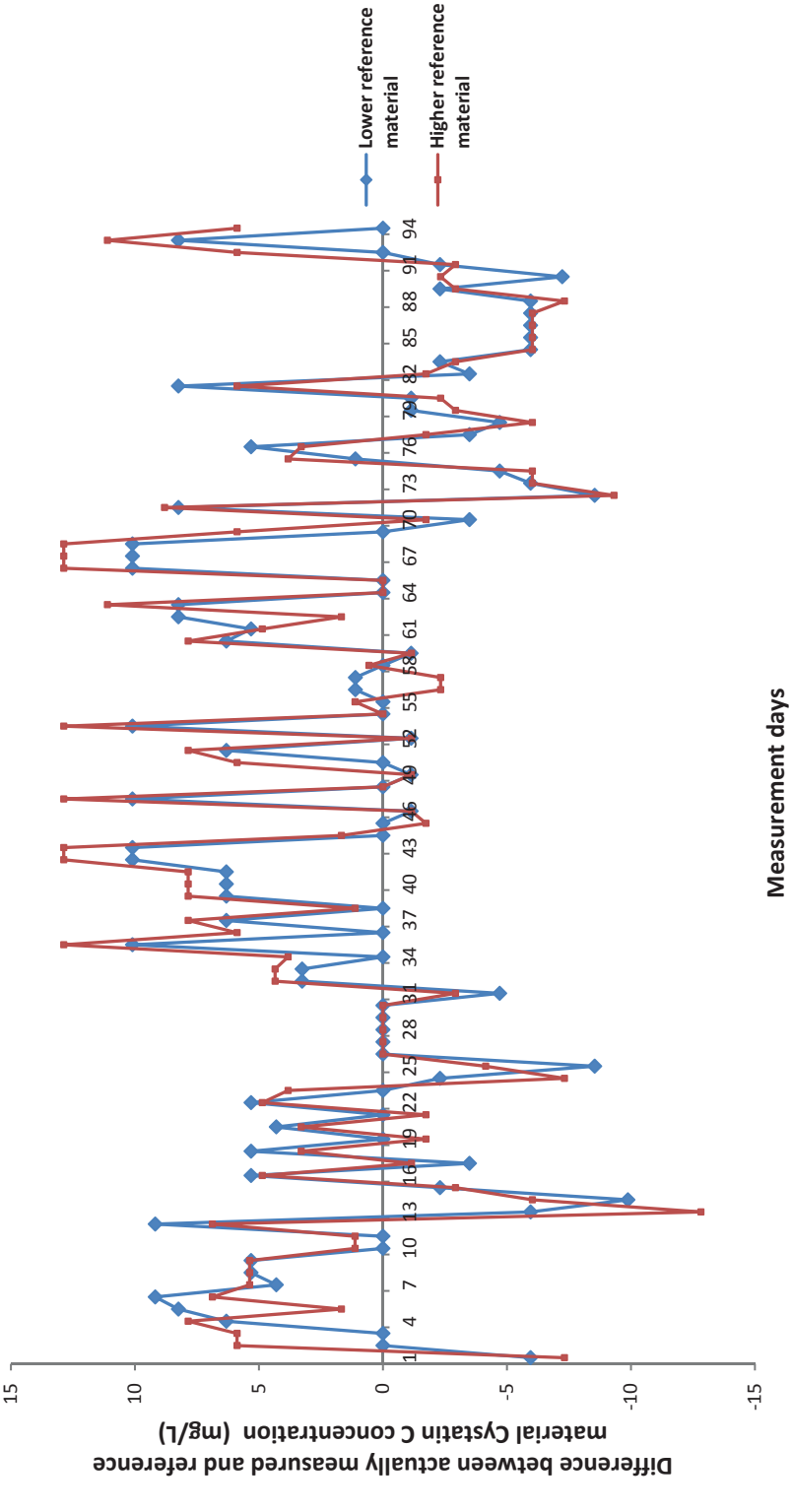
Appendix Figure 1 | **Drift and Day to day variability in cystatin C measurements**

A | **In lower reference material**
(Red line indicates lower reference material and blue line indicate variation in actually measured cystatin C)



B | **In higher reference material**
(Red line indicates higher reference material and blue line indicate variation in actually measured cystatin C)





Appendix Figure 2 | **Difference between actually measured and reference material cystatin C concentrations across measurement days**

Appendix Figure 3 | **Regression plot; difference between actually measured and lower reference material cystatin C concentration vs. difference between actually measured and higher reference material cystatin C concentration**